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BACTERIAL CARBOHYDRATE METABOLISM

NR 135-148; Nonr - 739 (00)

Annual Report

1 March 1952 - 28 February 1953

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TITLE OF PROJECT AND OBJECTIVES

Comparative biochemical study of the intermediary carbohydrate metabolism of Clostridium perfringens (BP6K), a strict anaerobe, and Bacillus subtilis (Marburg), a strict aerobe; elucidation of the mechanisms of fermentation and respiration in both organisms; characterization of the effect of nutrition on both types of metabolic mechanisms.

INTRODUCTION

Two progress reports have been submitted to date: Report No. 1, covering the period 1 March - 1 June 1952; and Report No. 2, covering the period 1 June - 1 September 1952. This report will cover the work completed during the contract year, 1 March 1952 - 28 February 1953.

PERSONNEL

In addition to the principal investigator (R. C. Bard), a full-time research assistant has been employed. From 1 February - 15 August 1952, the assistant's position was held by J. K. Martin, B.S., M.S., and from 1 September 1952 to date, the position has been held by M. L. Mallin, B.S., M.S. Pursuing related problems have been two graduate students, one working with C. perfringens (K. Shankar, B.S., A.M.) and one with B. subtilis (R. E. Klausmeier, A.B.).

The initial radioisotope data described in this report were obtained by the principal investigator while conducting research as a Research Collaborator in the laboratory of Dr. Martin Gibbs, Biology Department, Brookhaven National Laboratory, Upton, New York, from 16 June to 1 September 1952. In addition, active collaboration has been maintained with Dr. Gibbs who, in association with Dr. Lynn Paege of his laboratory, has continued study of the problem employing variously labeled glucose-C^{14}.

A comment concerning personnel is appended here. The two research assistants employed in this project have been, in the career sense, students. For example,

Mr. Martin left in the middle of August to return to his native country (New Zealand) to continue his studies with Dr. Edson at the University of Otago. The training received by Mr. Martin while an assistant in this project will qualify him further for independent research. Mr. Mallin has converted, since 1 February 1953, to a part-time graduate student basis, still functioning however as a full-time assistant by working overtime. Support during the summer months has been extended to Mr. Shankar, one of the graduate students mentioned above. It is apparent, therefore, that optimum use has been made of employment funds since the training of three advanced students has been expanded, a usage of funds in consonance with the policy of the Office of Naval Research to sponsor basic research. The principal investigator, however, has received no financial support from this contract, other than travel funds for attending meetings.

RESULTS

The results obtained are presented below following a description of the materials and methods employed. The results, moreover, are presented under three headings for reasons which will become apparent, and each section is prefaced by a brief historical resume introducing the phase of investigation being reported.

Section 1. Materials and Methods

Organisms. Clostridium perfringens, strain BP6K, and Bacillus subtilis, Marburg, strain C4.

Media.

Heart Infusion Broth (HIB): 1% tryptone, 1% yeast extract, 0.5% K_2HPO_4, 50% heart infusion, 50% water, desiccated heart particles, 1% glucose (sterilized by filtration and added aseptically to the previous mixture which had been heated at 120 C for 20 minutes). This medium is suitable for maintenance of C. perfringens in stock (stable for about 2 months at 4 C) and for preparation of the inoculum.

Medium A: same as HIB less heart infusion and heart particles. This medium was used to cultivate cells for metabolic studies with C. perfringens (not used in magnesium-deficiency study).

Evans' Peptone Medium: the method of Webb (1948) was used to prepare this medium in the magnesium-deficient state.

Complex (C) and Simple (S) media: these media are described by Gary and Bard (1952a).

Temperatures: incubation of cultures and manometric studies were conducted at 37 C.

Chemical Methods. To determine glucose, acetylmethylcarbinol, butanediol and ethanol, the methods of Neish (1950) were employed. Volatile acids were separated and measured by the celite method of Bueding and Yale (1951). Lactic acid was measured according to Barker and Summerson (1941), pyruvic acid according to Friedemann and Haugen (1943) and manometric gas exchanges according to Umbreit et al. (1949). Carbon monoxide preparation and use are described by Gary and Bard (1952a).

Section 2. Glucose fermentation and respiration by C. perfringens.

Glucose fermentation by this organism has been studied most recently by Pappenheimer and Shaskan (1944) and Bard (1949; Bard and Gunsalus, 1950), although the metabolic pathway remains to be described. Glucose respiration has been studied by Bard (1949, 1952), the general results being: Q_{O_2}, 100-180; Q_{CO_2}, 150-200; RQ, 1.6-2.0; per mole of glucose, 0.7-1.1 moles O_2 consumed, 1.4-1.8 moles CO_2 formed.

From these experiences, it was known that the history of the inoculum and the nature of the culture medium play significant roles in the fermentative and respiratory patterns of the cells obtained. Respiratory balances were not available and other data (inhibitors, radioisotopes, etc.) were either lacking

or of a random nature. Accordingly, a rather systematic investigation was launched, using resting cell suspensions to determine the pathways of glucose fermentation and respiration.

Inoculum: As a result of studies summarized below, involving the use of inocula from several sources and of various ages, the following inoculation procedure was adopted: daily passage through HIB, using 1% inoculum from a previous 12 hours old HIB culture, all HIB cultures being incubated for 12 hours; to obtain cells for metabolic study, Medium A was inoculated with a 1% inoculum from a 12 hours old HIB culture, the Medium A culture being harvested after 5-6 hours incubation. This procedure assures resting cells with high fermentative and respiratory activities.

The conditions required for high respiratory activity are (1) daily transfer of HIB cultures, incubating for 12 hours at 37 C; (2) the HIB cultures must be used immediately as the inoculum since an intermediate period of storage, even at 4 C, yields Medium A cultures of varying activity; (3) repeated pre-passage through Medium A before inoculation into Medium A gives the same effect. It appears that the heart infusion and/or heart particles in HIB contain a factor(s) necessary for vigorous respiratory activity.

Fermentative activity is much less sensitive to inoculation variations although use of a very old HIB culture (12-15 days storage at 4 C after 12 hours incubation at 37 C) as inoculum yields Medium A cultures whose cells are incapable of gaseous fermentation in vitro; a homolactic fermentation may have occurred under these conditions although this possibility was not tested. Such cells are also incapable of respiration.

Effect of glucose: Growth occurs in Medium A even in the absence of added glucose. Cells harvested from such medium lacking glucose possess practically no respiratory activity although fermentative capacity remains unimpaired. These results may be interpreted as indicating that either the aerobic system is "adaptive" (requires glucose) or that the aerobic glucose dissimilatory mechanism,

probably with reference to H-transport, is different from the anaerobic mechanism.

Effect of oxygen: Variation of the atmosphere over Medium A cultures, containing glucose, does not affect either the fermentative or the respiratory metabolism of resting cells; 100% oxygen does not inhibit growth in Medium A. These results indicate that a suitably low oxidation-reduction potential is achieved by the combination of medium, inoculum, and inoculation procedure used so that growth is not inhibited even in an atmosphere of pure oxygen. Also, it may be concluded that the respiratory system is constitutive (provided glucose is supplied: see above), requiring no oxygen for its elaboration during growth.

Age of cells: Fermentative and respiratory rates decrease with increasing age of cells, tested over the age range of 5-15 hours. Old cells' (15 hours) respiration is stimulated by washing the cells with saline although even greater stimulation is obtained if a boiled cell extract is added. These results suggest that (1) washing removes an inhibitor(s) from old cells (acids?) and (2) washing removes a thermostable diffusible factor (coenzyme fragment?) which is present in cell-free extracts.

Stability of cell suspensions: Allowing cells to stand for 2 hours at room temperature does not appear to reduce respiration, and sometimes respiration is stimulated (washing effect?: see above). Even bubbling air for 2 hours through the cells suspension causes only a moderate decrease (30%) in respiratory rate although the extent of oxidation remains unimpaired.

Optimum pH for respiration: Over a pH range of 5.4 to 8.0, with 0.044 M potassium phosphate buffer, the optimum pH for respiration is 6.5. This pH and phosphate level was employed throughout all studies of fermentation and respiration.

Optimum dry weight of cells: The dry weight of cells used in manometric studies of fermentation and respiration was 3-5 mg. At this cell weight level, the respiratory rate is maximum. Amounts of cells less than 3 mg and more than 5 mg respire at slower rates but probably for different reasons. Use of few cells simply does not afford optimum enzyme level for the amount of glucose used (10 μM). Use of excessive cell masses results in hydrogen evolution, a situation which may be termed "semi-fermentation." Evidence for this phenomenon is afforded by the observation that with excessive cell masses O_2 uptake occurs rapidly for the first 5-15 minutes after which O_2 uptake ceases or actual gas production ensues for about 5-15 minutes after which O_2 uptake ceases or actual gas production ensues for about 5-15 minutes, the gas probably being H_2 since any CO_2 formed is absorbed by the strong alkali present in the flask. Accordingly, less O_2 is required to oxidize the hydrogen carried by the H-transport system and, moreover, a situation obtains which forbids accurate calculation of the Q_{O_2}. Attempts to eliminate the H_2- burst by addition of palladium - asbestos failed since the reaction between H_2 and pallidium is too slow to prevent H_2 evolution. Even if these attempts were successful, however, an artifact would be introduced since in addition to having O_2 available as the H-acceptor, palladium would also accept H_2, a situation leading to no amelioration of the calculation problem. Hence, it was decided best simply to avoid the H_2-burst by employing a cell mass which appears not to engage in "semi-fermentation."

Dissimilatory balances: Using 5 hours old cells grown in Medium A with a 1% inoculum of 12 hours old HIB cells -- the routine inoculation and cultivation procedures employed -- balances such as follow were obtained; these are representative of others performed.

Per mole of glucose dissimilated	Anaerobic (fermentation)	Aerobic (respiration)
H_2 ($Q = 150$)	1.75	--
O_2 ($Q = 130$)	--	0.75
CO_2 ($Q^{H2} = 60$; $Q^{O2} = 150$)	1.97	1.83
CH_3CH_2OH	1.16	0.78
CH_3COOH	0.60	1.03
$CH_3(CH_2)_2COOH$	0.12	0.14
$CH_3CHOHCOOH$	0.06	0.12
C-recovery	103%	105%
O/R	0.92	1.08
Obs. CO_2/Calc. CO_2	0.99	0.88

The outstanding differences between these balances are, of course, the utilization of O_2 at the expense of H_2 and the lower level of ethanol during respiration with an increase in acetic acid. Under the conditions described, slight quantitative variations were noted but these do not merit special comment. To be noted is that the alcohol is ethanol; no butanol was found.

Occasionally, small amounts of pyruvic acid were detected during both fermentation and respiration. Accumulation of pyruvic acid reaches significant proportions (0.5-0.8 mole per mole of glucose dissimilated), however, when older cells (14 hours old) are employed for the resting cell suspension in respiration studies. Some (about 0.2 mole) pyruvic acid is found even during fermentation under these conditions. It would appear from these observations that pyruvic acid is either an intermediate of glucose dissimilation or a product of such an intermediate. On occasion, a substance yielding a 2,4-dinitrophenylhydrazone of methylglyoxal was detected (less than 0.1 mole), raising the possibility that methylglyoxal may also be an intermediate or derived therefrom.

Inhibitors: A rather systematic study of the effect of those inhibitors reported as effective in glucose dissimilatory mechanisms of other organisms was made. The inhibitor was mixed with the cells and phosphate (0.04 M) buffer prior to the addition of glucose; the time inhibitor and cells were in contact before substrate was added was 30-45 minutes.

Concentrations of sodium azide of 0.01 M or higher are required to inhibit markedly the rates and extents of fermentation and respiration. Growth is not inhibited by 0.01 M azide, requiring 0.1 M for inhibition. 2,4-Dinitrophenol, at concentrations of 0.01 M or higher, inhibits markedly the rates and extents of fermentation and respiration; fermentative hydrogen production, however, is more sensitive, being affected at concentrations lower than those required to exert a similar effect on the remaining reactions. These data suggest that the inhibitions observed may be related to phosphorylation mechanisms operative during fermentation and respiration. The high levels of these inhibitors required, however, make such interpretation speculative. To be noted here is the fact that addition of inorganic orghophosphate during fermentation and respiration stimulates the rates of those reactions (see below), suggesting participation of inorganic phosphate in glucose dissimilation which probably leads to the formation of organic and high energy phosphate compounds. Until specific data are obtained, however, this issue remains unclear.

The metal chelating agent, α, α'-dipyridyl, at levels of 0.001 - 0.003 M, totally inhibits both fermentation and respiration. This finding has been reported previously (Bard and Gunsalus, 1950) with iron-deficient C. perfringens. The site of inhibition may safely be assumed to be the metallo-aldolase found in this organism.

Iodoacetate, at the level of 0.0005 M, completely inhibits both fermentation and respiration, indicating merely participation of a sulfhydryl type enzyme. Since glyceraldehyde phosphate dehydrogenase has been demonstrated in iron-deficient C. perfringens (Bard, 1949), a sulfhydryl type enzyme, it is probably

that this oxidative enzyme participates in fermentation and respiration. It is also possible that the aldolase of this organism is inhibited by iodoacetate since a reducing agent is required for optimum activity of this enzyme (Bard and Gunsalus, 1950).

Potassium cyanide, at levels of 0.001 M or less, does not affect fermentation. Respiration, however, is inhibited by 0.001 M cyanide although only the rate of respiration is affected and not the final extent. Carbon monoxide (95% CO/5% O_2) does not affect respiration. Fermentation, however, is affected by carbon monoxide (95% CO/5% N_2), gas production being inhibited and the fermentation being shifted to a homolactic type. Stephenson (1949) cites the work of Bacon which is in agreement with the CO inhibition of gaseous fermentation by this organism. In the experiments being reported here, visible light does not reverse the CO inhibition at 37 C. The data with cyanide and CO, with reference to respiration, permit the conclusion that the mechanism of respiration is probably not heme-like in nature. The CO inhibition of gaseous fermentation, however, is not as easily explained. It is possible that a heme type enzyme is involved in pyruvate dissimilation (it is assumed that pyruvate is an intermediate of fermentation), perhaps the hydrogenase catalyzing the formation of gaseous hydrogen from atomic hydrogen.

The results with sodium fluoride are numerous, varied and somewhat unexpected. The data, with remarks, are summarized in table 1. A discussion of the effects of this inhibitor is postponed until after additional data are presented below. It was found, also, using iron-deficient cells prepared according to Bard and Gunsalus (1950), that the homolactic fermentation obtained under these conditions is almost totally inhibited by 0.05 M sodium fluoride, even in the presence of phosphate; phosphate also stimulates this homolactic fermentation.

The effect of various levels of arsenate on fermentation and respiration was tested; the data are presented in table 2.

Table 1

Effect of sodium fluoride on glucose fermentation and respiration

Inorg. PO_4	NaF	Mg^{++}	Fermentation			Respiration		Remarks
			Glucose uptake	H_2 prod.	CO_2 prod.	O_2 uptake	CO_2 prod.	
Molarity			Per cent inhibition					
0	0	0	0	~ 0	~100	~ 0	~0	1
0.015 to 0.132	0	0 or 0.001	0	0	0	0	0	2
0	0.005 to 0.05	0 or 0.001	0 to ~ 100	0 to 50-65	~100	0 to 100	0 to 100	3
0.044	0.01 to 0.05	0 or 0.001	--	0	0	0	0	

1: Although extents are not affected, rates are decreased significantly. Fermentative CO_2: none produced for about 2 hrs when some is formed but extent is low.

2: Normal values obtained in all cases, apparently 0.015 M inorganic PO_4 sufficing for optimal rates. 0.044 M inorganic PO_4 used in all experiments unless otherwise indicated.

3: Since inorganic PO_4 is absent, no fermentative CO_2 is formed (see 1, above). When NaF is added to flasks without KOH in center well, glucose uptake and H_2 formation is proportional to NaF level. Also, as NaF level is increased, almost twice as much lactic acid is formed per mole of glucose fermented. 2,4-dinitrophenylhydrazone of methylglyoxal identified spectrophotometrically in these flasks. In flasks without KOH, traces of pyruvic acid found but no methylglyoxal.

Table 2

Effect of arsenate on glucose fermentation and respiration*

$AsO_4^{\equiv}$ conc.	Fermentation				Respiration			
	H_2		CO_2		O_2		CO_2	
M	Q	µM	Q	µM	Q	µM	Q	µM
0	176	20.2	105	13.9	118	14.4	95	14.0
0.005	200	18.8	--	--	45	9.0	25	5.0
0.01	156	17.5	104	11.0	26	9.0	20	5.9
0.05	75	12.5	27	6.8	2	0.9	1	0.2

* In the presence of the standard concentration of inorganic phosphate (0.044 M).

It is clear from these data that arsenate inhibits strongly both fermentation and respiration. It appears that arsenate competes with phosphate for substrate (intermediates) leading to arseno complexes which are subject to arsenolysis and hence leading to uncoupling of phosphorylation. These data add support to the suggestion (see above) that both anaerobic and aerobic glucose dissimilation involve esterification of inorganic phosphate.

Arsenite, at levels ranging from 0.005 - 0.05 M, almost completely inhibits glucose fermentation but has practically no effect on respiration. The significance of these findings is not clear. It can be suggested that the inhibition of fermentation by arsenite indicates the participation of pyruvate as a metabolic intermediate. However, the absence of arsenite inhibition during respiration does not permit generalization of this interpretation to cover a common glucose dissimilatory pathway under both anaerobic and aerobic conditions.

Pyruvate metabolism: Using 20 µM sodium pyruvate as substrate instead of glucose, a brief manometric study of pyruvate metabolism was made. It was found that inorganic phosphate (0.04 M, pH 6.5) stimulates the rates of H_2 and CO_2 evolution; about 1 mole each of H_2 and CO_2 are formed per mole of pyruvate fermented. The rates of H_2 and CO_2 evolution with pyruvate as substrate are about ½ - 1/3 those obtained with glucose as substrate. Addition of 0.02 - 0.05 M sodium fluoride to this system containing inorganic phosphate has little inhibitory effect on pyruvate fermentation. However, addition of fluoride in the absence of phosphate causes about 50% inhibition of pyruvate metabolism, an effect qualitatively similar to the finding during glucose fermentation. The inhibited pyruvate fermentation is not directed toward a lactic type fermentation since there is no increase in lactic acid formation.

Other substrates: Neither young (5 hours old) nor old (14 hours old) cells, grown in the presence of glucose, respire in the presence of mannitol, sorbitol, galactose, gluconic acid, glucuronolactone, glucuronic acid, ribose, xylose or arabinose. Cells grown in the presence of glucuronolactone or glucuronic acid with without glucose do not respire in the presence of glucuronolactone or glucuronic acid. Slight respiratory activity was noted with sucrose and lactose. With fructose and maltose, respiration occurs but only between 1/5 to 1/2 the rate noted with glucose.

Radioisotope data: Using glucose-1-C^{14} as substrate, it was found that none of the CO_2 formed during fermentation and respiration contains C^{14}. The methyl carbon of the acetic acid from such reaction mixtures contains C^{14}, the carboxyl carbon being unlabeled.

Using glucose-3,4-C^{14}, only 50-65% of the C^{14} was isolated in the CO_2 formed during fermentation and respiration. Location of the remaining label remains to be determined. The specific radioactivity of the glucose-3, 4-C^{14} has been checked with fermenting yeast and the calculated activity appears to be correct.

Using glucose-6-C^{14}, the CO^2 derived therefrom during fermentation contains no C^{14}.

These incomplete data serve to suggest that this organism possesses a pathway of glucose dissimilation unlike those previously described. The actual difference may prove to consist of a variation of a known mechanism.

Discussion: All the data obtained thus far do not support the conclusion that the mechanism of glucose dissimilation present in C. perfringens is identical to a known mechanism. Two main objections to this conclusion are presented: (1) fluoride insensitivity; (2) the radioisotope data. The fluoride data with glucose may be interpreted as paralleling those with pyruvate, hence focusing attention upon pyruvate dissimilation as the site of fluoride inhibition in the absence of phosphate. If this is true, then it must be concluded that an enolase-type enzyme is not present in this organism, a possible interpretation in view of the data presented above and the findings of Stone and Workman (1937). Moreover, the peculair isotope data need interpretation. At this point, it can only be concluded that a variation of a known mechanism may be operative. Until enzymatic data are available, a reasonable interpretation is not apparent.

Section 3. Metabolic aspects of magnesium deficiency in C. perfringens (K. Shankar

It has been reported (Shankar and Bard, 1952) that Mg^{++} deficiency during growth leads to elongated cell formation. Such cells ferment glucose with the same rates and extents of H_2 and CO_2 production as do Mg^{++}-sufficient cells. Growth of Mg^{++}-deficient cells in the presence of added Co^{++} is considerably impeded and such cells are then incapable of gaseous fermentation although still able to ferment glucose to lactic acid. Such data suggest that Co^{++} interferes with the synthesis of the enzyme system responsible for H_2 and CO_2 formation.

Another method (Webb, 1948) has been used to obtain Mg^{++}-deficient medium. With medium so treated, cell morphology is even more markedly affected. Very long cellular filaments are obtained instead of merely elongated cells. It was decided

to study the glucose metabolism of the filamentous cells rather than the elongated cells since the biochemical "lesion" involved may be expected to be more extensive and more readily descernible in the filamentous cells.

It was found that the Webb method of preparing Mg^{++}-deficient medium works only with Evans peptone, a British product, and not at all with the numerous other peptone and tryptone media tested. Attempts to elucidate the basis for this difference yielded no specific relationship. However, use of Evans peptone has been made to obtain the filamentous, Mg^{++}-deficient cells.

The rate of glucose fermentation of Mg^{++}-deficient, filamentous cells is about 1/8 that found with Mg^{++}-sufficient, normal cells. Chemical analysis reveals that glucose uptake by Mg^{++}- deficient cells is markedly reduced per unit time, suggesting a primary biochemical weakness in such cells. The glucose is in time totally fermented although the time required is about tenfold in length that found with Mg^{++}-sufficient cells.

Section 4. Carbohydrate metabolism of Bacillus subtilis (R. E. Klausmeier)

It has been reported (Gary and Bard, 1953a, b) that growth of B. subtilis in inorganic nitrogen, glucose, salts medium (Simple medium, yielding S-cells) results in cells virtually incapable of glucose fermentation whereas growth in tryptone, yeast extract, glucose medium (Complex medium, yielding C-cells) results in cells capable of vigorous homolactic fermentation of glucose. Moreover, an enzymatic deficiency apparently responsible for the lack of the fermentative capacity in S-cells was found to be linked to the absence of glyceraldehyde phosphate dehydrogenase in such cells.

Further study has revealed that addition of H-acceptors (acetylmethylcarbinol, diacetyl, pyruvic acid, tetrazolium salts, hydroxylamine) to S-cells during anaerobic glucose dissimilation leads to a vigorous fermentation of the glucose. Using acetylmethylcarbinol as H-acceptor and examining the role of this compound during fermentation reveals that the H-acceptor is reduced to butanediol

(hence proving that it is acting as a H-acceptor) while the glucose is dissimilated to lactic acid, acetylmethylcarbinol and CO_2.

Plans for Future

(1) Initiate enzymatic studies of the mechanism of glucose fermentation in C. perfringens, both of Mg^{++}-deficient and -sufficient cells, and in B. subtilis.

(2) Continue collaboration with Dr. Martin Gibbs on radioisotope studies.

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